REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
2005	open literature publication	

4. TITLE AND SUBTITLE

Genomic Analysis of Rodent Pulmonary Tissue Following Bis-(2-chloroethyl) sulfide Exposure

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

USAMRICD-P04-027

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Institute of Chemical Defense
ATTN: MCMR-CDA-T
3100 Ricketts Point Road
Aberdeen Proving Ground, MD
21010-5400

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

Published in Chemical Research Toxicology, 18(1), 28-34, 2005

14. ABSTRACT

See reprint

15. SUBJECT TERMS

Sulfur mustard, gene expression, chemical warfare agent, medical countermeasues, lung tissue, inhalation, DNA damage

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			James F. Dillman III
UNLIMITED	UNLIMITED	UNLIMITED	UNLIMITED	7	19b. TELEPHONE NUMBER *(include area code)* 410-436-1723

Chem. Res. Toxicol. **2005,** *18,* 28−34

Chemical Profiles

Genomic Analysis of Rodent Pulmonary Tissue Following Bis-(2-chloroethyl) Sulfide Exposure

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Received September 10, 2004

Bis-(2-chloroethyl) sulfide (sulfur mustard, SM) is a carcinogenic alkylating agent that has been utilized as a chemical warfare agent. To understand the mechanism of SM-induced lung injury, we analyzed global changes in gene expression in a rat lung SM exposure model. Rats were injected in the femoral vein with liquid SM, which circulates directly to the pulmonary vein and then to the lung. Rats were exposed to 1, 3, or 6 mg/kg of SM, and lungs were harvested at 0.5, 1, 3, 6, and 24 h postinjection. Three biological replicates were used for each time point and dose tested. RNA was extracted from the lungs and used as the starting material for the probing of replicate oligonucleotide microarrays. The gene expression data were analyzed using principal component analysis and two-way analysis of variance to identify the genes most significantly changed across time and dose. These genes were ranked by p value and categorized based on molecular function and biological process. Computer-based data mining algorithms revealed several biological processes affected by SM exposure, including protein catabolism, apoptosis, and glycolysis. Several genes that are significantly upregulated in a dose-dependent fashion have been reported as p53 responsive genes, suggesting that cell cycle regulation and p53 activation are involved in the response to SM exposure in the lung. Thus, SM exposure induces transcriptional changes that reveal the cellular response to this potent alkylating agent.

Introduction

Bis-(2-chloroethyl) sulfide (sulfur mustard, SM)[1-3] is a carcinogen and chemical warfare agent that was used on the battlefield during World War I and has since been used in several conflicts around the globe. SM exposure results in cutaneous, pulmonary, and ocular injury. Despite much research, an effective medical countermeasure for SM exposure has not been developed because the molecular mechanism of SM toxicity is not well-understood. SM is a potent alkylating agent capable of covalently modifying cellular macromolecules such as DNA and protein by nucleophilic interaction (*1*). Because it is a bifunctional molecule, SM has the capacity to cross-link biological molecules with which it reacts. Covalent modification of DNA by SM has been well-characterized due to the use of SM and related molecules as anticancer therapies (*1*), and covalent modification of proteins by SM has also been demonstrated (*2*−*5*). A variety of molecular targets and pathways have been implicated in the mechanism of toxicity of SM exposure (*1*); however, the precise mechanisms of cellular injury remain undelineated.

The lung is a primary target of SM vapor. Inhalation exposure causes pulmonary edema and toxicity to bronchial epithelial cells that can potentially lead to bronchopneumonia and death (*1*). Because SM is viscous and highly reactive, experiments designed to investigate SM toxicity by inhalation exposure are highly problematic due to the potential for accidental exposure and the difficulty in decontaminating equipment and supplies. Furthermore, SM is unstable in biological fluids, making it difficult to quantify the dose received in the lungs after an inhalation exposure. Therefore, attempts have been made to investigate SM-induced lung injury by using alternative exposure routes including subcutaneous or intravenous injection (*6*, *7*). In subcutaneous and intravascular models, the lung has been shown to be one of the major target organs for SM accumulation. Kinetic studies using ^{14}C-labeled SM by Boursnell et al. (*8*) and more recently by Maisonneuve et al. (*9*, *10*) demonstrated

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[1] Abbreviations: SM, sulfur mustard; Q-PCR, quantitative real-time polymerase chain reaction; IPA, isopropyl alcohol; PCA, principal component analysis.

[2] The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

[3] The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE1888.

10.1021/tx049745z CCC: $30.25
Published on Web 12/24/2004

that radioactivity accumulates in the lung within 2–4 h after administration and that the lung is a major site for the deposition of radioactivity. Langenberg et al. (*11*) have shown by immunoassay that i.v. administration of SM in the guinea pig induces significant DNA adduct formation in the lung within 3 min of an i.v. challenge. Thus, alternative routes of administration have been used to create a SM lung exposure.

To better delineate the molecular mechanisms of SM toxic effects and to identify potential therapeutic targets for the development of effective medical countermeasures, we performed dose–response and time–course studies of rat lung exposed to SM and assayed global changes in gene expression using oligonucleotide microarrays. By developing a robust data set (three biological samples for each treatment, each assayed in duplicate), we were able to identify statistically significant changes in gene expression due to SM exposure. From these significant gene expression changes, we identified specific cellular pathways that were modulated by SM exposure. These results will aid in the identification of potential therapeutic targets and will direct future work in the development of medical countermeasures to alleviate the effects of SM exposure.

Experimental Procedures

Rat Lung Exposure Model. Male Sprague–Dawley rats (250–300 g; Charles River, Wilmington, MA) were anesthetized with sodium pentobarbital (5 mg/100 g body weight, i.p.). A small incision in the superficial femoral area was performed to expose the femoral artery and vein. Vehicle (isopropyl alcohol, IPA) or SM (1, 3, or 6 mg/kg) delivered in vehicle was then administered i.v. into the femoral vein with a sterile tuberculin syringe at 1 mL/kg at a rate of 50 μL/min. A matched group of rats were injected in a similar manner with sterile physiological saline. At 0.5, 1, 3, 6, or 24 h after SM administration, rats were euthanized by exposure to a 100% CO_2 gas environment. Following expiration, a tracheotomy was performed using a stainless steel cannula. The pulmonary artery was isolated and cannulated, and a small incision was made in the left atrium. The rats were ventilated with 3 mL of tidal volume at 60 breaths/min. During ventilation, 30 mL of iced saline was infused over a 3–5 min period into the pulmonary artery to wash all blood from the lung. Blanched lungs were removed and immediately placed in liquid nitrogen and stored at −80 °C. In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources, National Research Council, in accordance with the stipulations mandated for an AAALAC accredited facility.

Gene Expression Profiling. All experiments were performed using Affymetrix Rat RAE230A oligonucleotide arrays, as described at http://www.affymetrix.com/support/technical/datasheets/rat230_datasheet.pdf (Affymetrix, Santa Clara, CA). Frozen rat lungs were homogenized in Tri Reagent (Sigma-Aldrich Chemical Co., St. Louis, MO), and the total RNA was extracted according to the manufacturer's protocol (http://www.sigmaaldrich.com/sigma/bulletin/t9424bul.pdf). RNA was further purified using RNeasy columns (Qiagen, Valencia, CA). The quality and amount of RNA were monitored throughout processing with an Agilent Bioanalyzer (Agilent, Palo Alto, CA) and a NanoDrop ND-1000 UV–vis Spectrophotometer (Nanodrop Technologies, Rockland, DE). Purified RNA was used to prepare biotinylated target RNA, with minor modifications from the manufacturer's recommendations (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Briefly, 10 μg of total RNA was used to generate first strand cDNA by using a T7-linked oligo(dT) primer. After second strand synthesis, in vitro transcription was performed with biotinylated UTP and CTP (Enzo Kits from Affymetrix), resulting in approximately 100-fold amplification of cRNA. The target cRNA generated from each sample was processed as per manufacturer's recommendation using an Affymetrix GeneChip Instrument System (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Briefly, spiked controls were added to 15 μg of fragmented cRNA before overnight hybridization using 10 μg of cRNA. Arrays were then washed and stained with streptavidin–phycoerythrin before being scanned on an Agilent GeneArray Scanner. After scanning, array images were assessed by eye to confirm scanner alignment and the absence of significant bubbles or scratches on the chip surface. The 3′/5′ ratios for GAPDH were between 1 and 5.8 (eight of 154 chips that were scanned were above 3.0), and the ratios for β-actin were between 1.13 and 5.88 (19 of 154 chips that were scanned were above 3.0). BioB spike controls were found to be present on 133 out of 154 chips that were scanned (86.4%, two were called marginal and not included in the calculation), with BioC, BioD, and CreX also present in increasing intensity. When scaled to a target intensity of 150 (using Affymetrix Microarray Suite 5.0 array analysis software), scaling factors for all arrays were between 0.35 and 4.068.

Data Analysis. To develop a robust data set for subsequent statistical analysis, a sample size of $n = 3$ rats was used for each time and dose indicated ($n = 3$ biological replicates), and probes generated from each sample were used to probe duplicate chips ($n = 2$ chip replicates). Scanned output files from each array were inspected for quality control as described and then processed using Affymetrix Microarray Suite (v 5.0), Affymetrix MicroDB (v3.0), and Affymetrix Data Mining Tool (v 3.0) as described (http://www.affymetrix.com/index.affx). The raw data were imported as a comma separated values (.csv) file into Partek Pro 5.0 (Partek, St. Louis, MO). To obtain a data set with a normal distribution for statistical analysis, the raw data were normalized by the addition of a constant ($c = 1$) followed by log transformation (log base 2). The log-transformed data were analyzed by principal component analysis (PCA) to determine the significant sources of variability in the data. This information was used to determine grouping variables for ANOVA. A set of genes with significantly altered expression levels based on p values from the ANOVA was used to determine gene pathways and molecular networks significantly affected by SM treatment. Onto-Express was used to screen for significant pathways modulated by SM exposure (*12*).

Quantitative Real-Time Polymerase Chain Reaction (Q-PCR). Q-PCR was used to validate the expression levels of selected genes. It has been observed that the expression levels of traditionally used "housekeeping" genes (e.g., β-actin, GAPDH) are affected by SM exposure (Phillips, C. S., and Schlager, J. J. Unpublished observations), and thus, methods commonly used to analyze Q-PCR data that utilize a housekeeping gene as an internal standard would be inaccurate in our system. This has been observed in other systems, and as a result, various methods have been employed to analyze Q-PCR data (*13*). To address this problem, we developed a modified analysis method based on the comparative Ct (cycle threshold) method described previously in User Bulletin 2 (*14*). In the modified method, the RNA input was accurately quantitated by multiple measurements with a NanoDrop ND-1000 UV–vis Spectrophotometer (Nanodrop Technologies) to ensure that each reaction was comparable based on equal amounts of starting material. In addition, an exogenously spiked control (Taq-Man Exogenous Internal Positive Control, Applied Biosystems, Foster City, CA) was used as our internal reference gene to calculate the ΔCt for each sample assayed. The ΔΔCt was then calculated based on the ΔCt of the IPA vehicle control and the corresponding treated sample. The fold change in gene expression was calculated from the ΔΔCt as $2^{-\Delta\Delta Ct}$.

All Q-PCR was performed using Taq-Man PCR reagents and analyzed using the ABI 7700 Sequence Detection System (Applied Biosystems). Primers and probes used for Q-PCR are listed in the Supporting Information (Table 1) and were designed

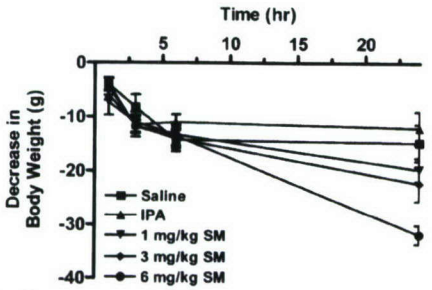

Figure 1. Rats exposed to SM intravenously lose body mass after 24 h. Rats were exposed to SM by i.v. injection. Weights were taken before injection and at the time of sacrifice at the indicated time points. A sample size of $n = 3$ for each time point and dose was used. Error bars represent standard deviation.

using ABI Prism Primer Express V1.0 (Applied Biosystems). To confirm the amplification specificity from each primer pair, the amplified PCR products were size-fractioned by electrophoresis in a 2% agarose gel in Tris borate ethylenediamine tetracetic acid buffer and photographed after staining with ethidium bromide. Only the expected products at the correct molecular size were identified. Total RNA was purified as described above and DNAse-I-treated on the purification column according to the manufacturer's protocol (Qiagen). The reverse transcription reaction was carried out using 5 μg of total RNA (final concentration 98 ng/μL) using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). A no-template control, a no-amplification control, and the experimental samples being tested (three biological replicates for each treatment group) run in triplicate (three technical replicates) were included for each gene analyzed. For each sample analyzed by Q-PCR, 300 nM primers and 250 nM probes were used as determined by preliminary optimization experiments for each probe–primer pair set. Amplification reactions were carried out using the instrument default cycle conditions.

Results

Intravenous Administration of SM Results in a Loss of Body Weight. The toxic effect of a single i.v. dose of SM is displayed by weight loss across both dose and time as shown in Figure 1. This was evident at 24 h when the higher SM doses of 3 and 6 mg/kg increased weight loss when compared with the IPA only treated rats at the same time point.

PCA. Gene expression data from SM-exposed rat lungs were analyzed by PCA (Supporting Information, Figure 1). PCA reduces the complexity of high-dimensional data and simplifies the task of identifying patterns and sources of variability in a large data set. The samples (three biological replicates each hybridized to two separate arrays) are represented by the points in the three-dimensional plot. The distance between any pair of points is related to the similarity between the two observations in high dimensional space. Samples that are near each other in the plot are similar in a large number of variables (i.e., expression level of individual genes). Conversely, samples that are far apart in the plot are different in a large number of variables. In this data set, the major sources of data variability are linked to time of exposure and SM dose. No significant data variability were attributed to sample processing, chip lot, or other technical parameters. The samples representing a 30 min SM exposure all partition together, regardless of the dose (Supporting Information, Figure 1). The 1, 3, 6, and 24 h samples all partition together with smaller clusters of each time point partitioning by dose. The 6 mg/kg dose at 6 h partitioned away from the other treatment groups. A small number of samples partitioned away from the main clusters (see top left of Supporting Information, Figure 1A). This may reflect biological variability among samples.

Statistical Analysis of Gene Expression Data and Identification of Genes Representing Modulated Biological Processes and Molecular Functions. The results of the PCA plot show that the major sources of data variability were due to time of exposure and dose of SM used. This information was used to design a two-way ANOVA using time of exposure and dose of SM as the grouping variables. The genes were ranked by p value based on this two-way ANOVA. Supporting Information, Table 2, reports the most significantly changed genes over time and dose at $p < 1 \times 10^{-20}$. To determine genes that represent molecular functions and biological processes that were most affected by SM exposure, the genes most significantly changed in expression level over time and dose ($p < 1 \times 10^{-9}$, 2500 genes) were mapped to the Gene Ontology (GO) (*15*). The GO project is a collaborative effort to address the need for consistent descriptions of gene products in different databases. A controlled GO vocabulary is maintained in a curated database. GO provides three structured networks of defined terms to describe gene product attributes. These are biological process, molecular function, and cellular compartment. We used the web-based search engine Onto-Express to map our genes to the GO database (*12*). Onto-Express translates lists of differentially regulated genes identified in high throughput gene expression experiments into functional profiles based on the GO. The statistical significance value is calculated, and results are displayed graphically as GO hierarchical trees. Supporting Information Table 3 summarizes genes that represent the molecular functions most affected by SM exposure ($p < 0.01$), and Supporting Information Table 4 summarizes the genes that represent the biological processes most affected by SM exposure ($p < 0.01$).

Genes Linked to p53 Response and Cell Cycle Regulation Are Modulated by SM Exposure. The most significantly changed gene was cyclin G (Supporting Information, Table 2), which shows a robust dose–response (Figure 2A). Cyclin G is a p53 responsive gene involved in cell cycle regulation. We found a number of other genes involved in cell cycle regulation that are significantly upregulated or downregulated in a dose–response manner by SM, including cyclin-dependent kinase inhibitor 1A (p21) (Figure 2B), mdm2 (Figure 2C), transducer of ErbB2 (TOB1) (Figure 2D), C-terminal binding protein (CtBP1) (Figure 2E), and Cut (Drosophilia)-like 1 (Cutl1) (Figure 2F). In addition, we mined from our data a number of genes that have been shown to be p53 responsive in other systems, including Eph2A (Figure 3A), metallothionein (Figure 3B), IEX1 (Figure 3C), serum inducible kinase (snk) (Figure 3D), Bax (Figure 3E), and ATF3 (Figure 3F).

Validation of Selected Microarray Results by Q-PCR. The reliability of our microarray data was confirmed using Q-PCR analysis of selected p53 responsive cell cycle regulators. As shown in Supporting Information Figure 2, the relative expression levels of cyclin G1, mdm2, and p21 based on Q-PCR analysis were consistent with the expression profiles determined by microarray analysis.

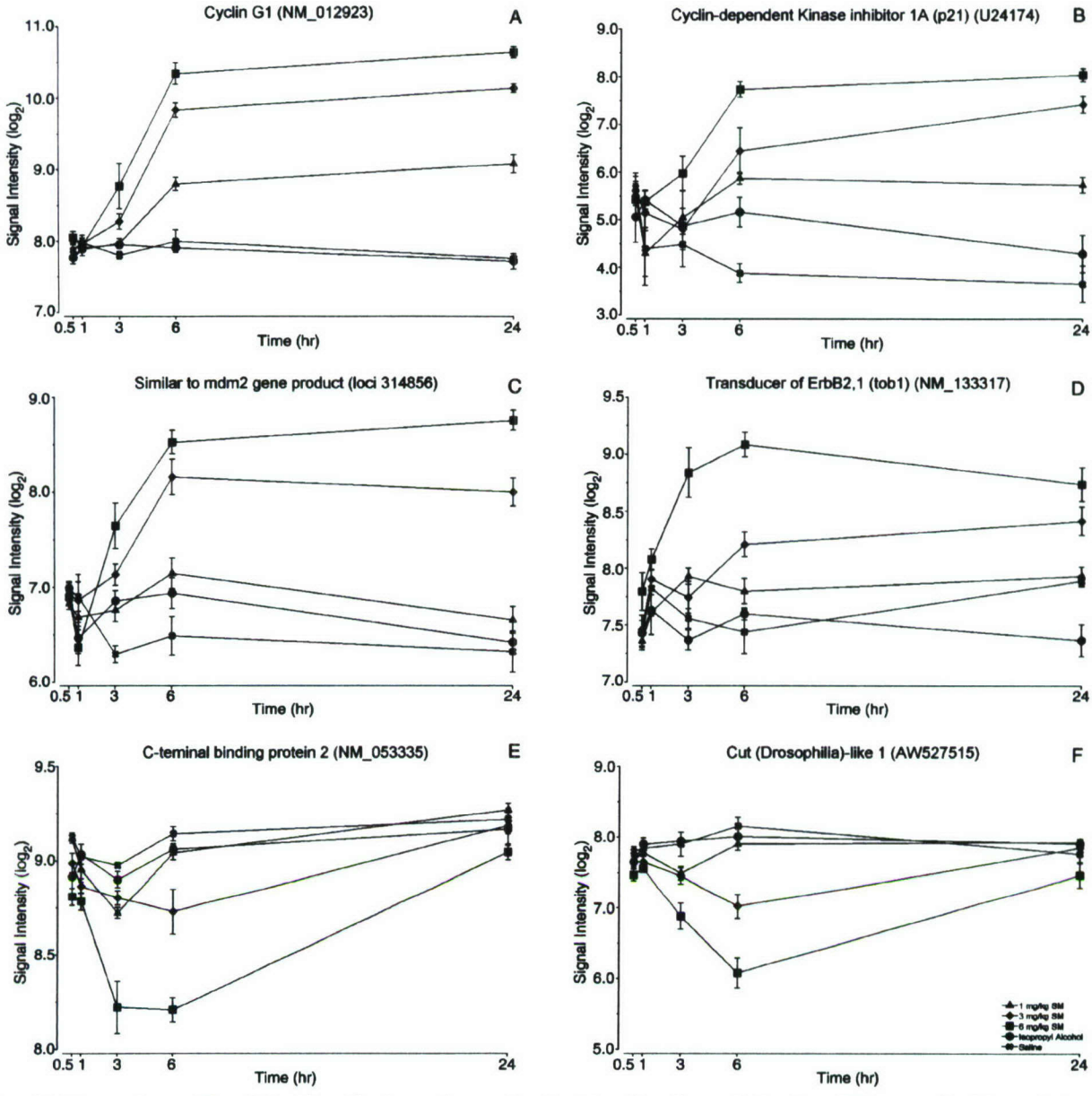

Figure 2. Expression profiles of significantly changed genes involved in cell cycle regulation from SM-exposed rat lung. A two-way plot of $\log_2$ expression level vs time for each treatment or SM dose was generated. ▲, 1 mg/kg SM; ◆, 3 mg/kg SM; ■, 6 mg/kg SM; ●, IPA; and ×, saline. Error bars represent standard deviation.

Discussion

An established model of lung injury induced by i.v. administration of SM was used to elucidate the effects of SM on rat lung. Our studies using this model show toxic effects and significant changes in lung gene expression resulting from a systemic exposure to SM. This is consistent with previous studies demonstrating that SM accumulates in the lung tissue (*8*−*10*) and can induce the rapid formation of SM−DNA adducts in the lung (*11*). As mentioned previously, wide-ranging cellular and molecular consequences have been described after exposure to SM both in vivo and in vitro in a variety of model systems and SM exposure paradigms. By utilizing microarray technology, we monitored the temporal and dose−response effects of SM exposure across a significant portion of the rat genome (~16K genes) and measured the effects of SM exposure in a variety of molecular systems.

It has been known for many years that one of the prominent effects of SM exposure is DNA damage due to SM-induced DNA cross-linking at the N-7 of guanine (*1*). With respect to the cellular response to DNA damage, it is apparent that there is a p53-mediated response to SM exposure. We base this conclusion on the fact that a number of the most significantly altered genes in SM-exposed rat lung are p53 responsive genes involved in cell cycle regulation, including cyclin G (*16*), p21 (*17*), and mdm2 (*18*). Transducer of ErbB2 (Tob) is a member of the Btg family of antiproliferative genes (*19*). Other members of this family have been shown to be p53 responsive genes (*20*, *21*). It has been shown previously that p53 protein levels increase following SM exposure (*19*, *22*, *23*). Using a microarray approach, we have been able to survey a large number of p53 responsive genes, including Eph2A (*24*), IEX1 (*25*), Bax (*26*), snk (*27*), ATF3 (*28*), and metallothionein (*29*). This adds strength to the

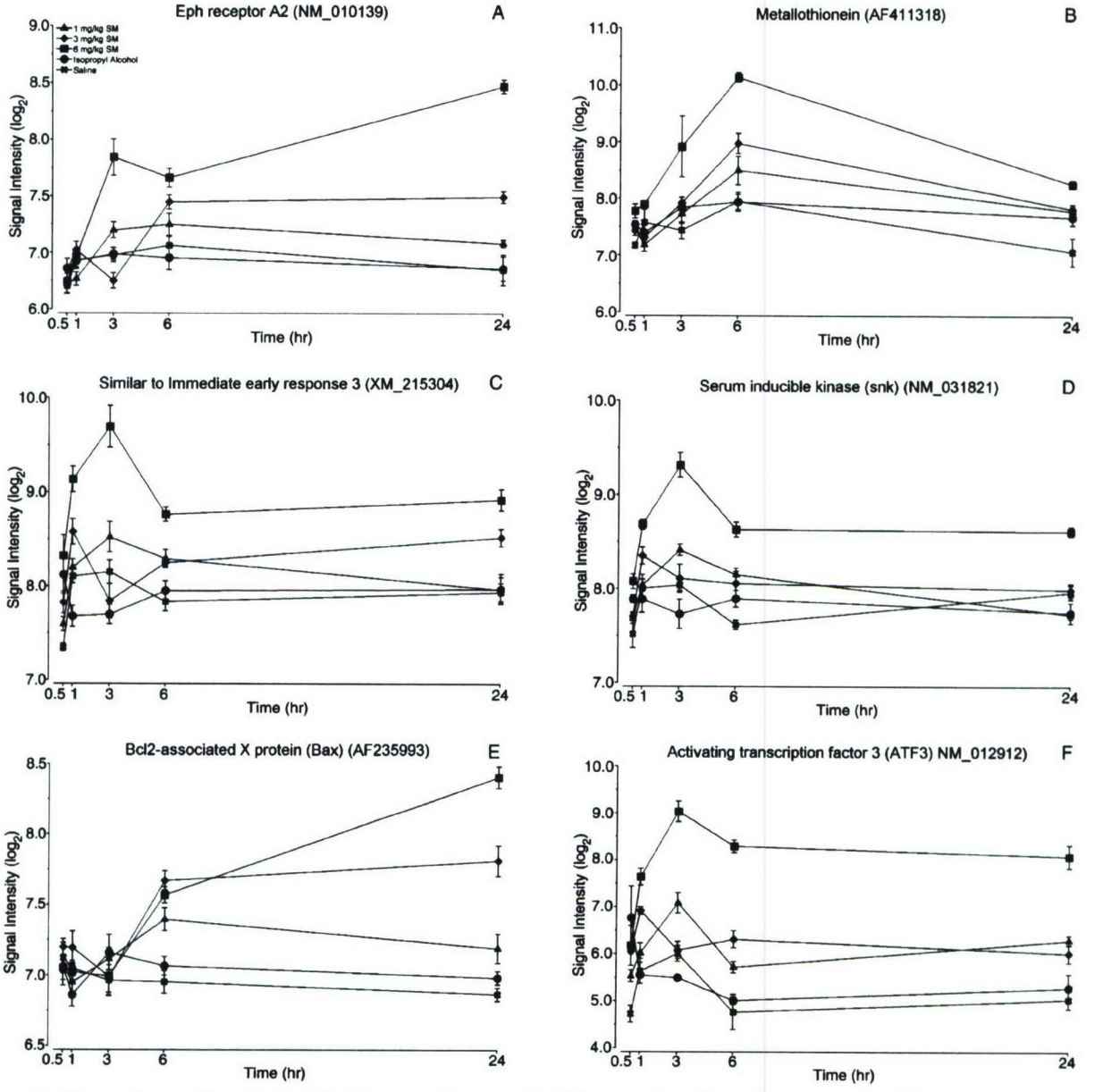

Figure 3. Expression profiles of selected p53 responsive genes in SM-exposed rat lung. A two-way plot of $\log_2$ expression level vs time for each treatment or SM dose was generated. p53 responsive genes involved in apoptosis (Eph2A, IEX1, and Bax), cellular injury response (metallothionein), mitosis (snk), and transcription (ATF3) are shown. ▲, 1 mg/kg SM; ◆, 3 mg/kg SM; ■, 6 mg/kg SM; ●, IPA; and ×, saline. Error bars represent standard deviation.

conclusion that there is a p53 response following SM exposure in rat lung. Figure 4 depicts an overall picture of the putative role of p53 in this particular model.

Increased levels of p21, a known inhibitor of cdk−cyclin complexes, can prevent cell cycle progression (*30*). Cyclin complexes are responsible for signaling the cell to move from the G1 phase through the restriction point and into the S phase. p21 blocks cell cycle progression in G1 by inhibiting cdk4/cyclinD and preventing phosphorylation of Rb and initiation of the S phase (*30*). Cut1-like (CutlL) has been shown to repress p21 expression in the S phase and can thereby overcome its inhibitory effects on cdk4/cyclinD (*31*). We show that p21 is upregulated in a dose-dependent manner and that Cut1L is downregulated in a dose-dependent manner (Figure 2). Thus, these gene expression changes are consistent with a cell cycle arrest at G1 after SM exposure.

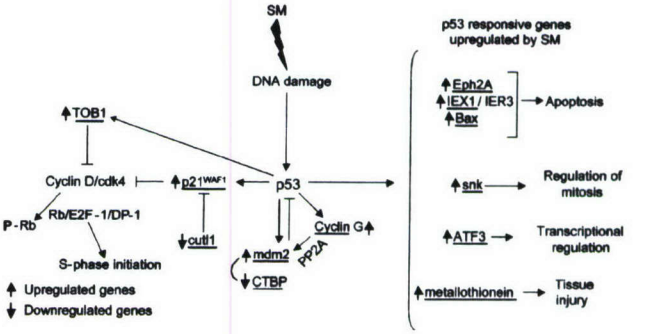

Figure 4. Gene expression data from SM-exposed rat lung reveals a potential p53 response. On the basis of gene expression data presented, the known genotoxic effects of SM, and work on p53 responsive genes, we propose that the p53 pathway is an important pathway activated by SM exposure in the rat lung.

Cyclin G1 and mdm2 are also upregulated after SM exposure (Figure 2). Both cyclin G1 and mdm2 are p53 responsive genes (*16*, *18*). Mdm2 forms a complex with p53 to block p53-mediated transactivation (*32*) and targets p53 for proteasome-dependent degradation (*33*). Cyclin G expression induces an mdm2-dependent decrease in p53 levels (*34*). Cyclin G has also been shown to be a regulatory component of the active protein phosphatase 2A holoenzyme, which activates mdm2 through dephosphorylation (*35*). Thus, cyclin G and mdm2 are likely involved in negative feedback regulation of p53 activity after SM exposure. Interestingly, the transcriptional repressor C-terminal binding protein 2 (CtBP2) is significantly downregulated in a dose–response manner after SM exposure (Figure 2). Microarray analyses of CtBP knockout and CtBP-rescued mouse embryo fibroblasts have revealed regulation of proapoptotic genes by CtBP, since the knockout cells showed higher expression of proapoptotic genes as compared with the rescued cells (*36*). In addition, it has been shown that the human homologue of mdm2, Hdm2, represses p53 activity through the recruitment of CtBP2 (*37*). Thus, downregulation of CtBP may act to increase expression of proapoptotic genes and increase the activity of p53. This may act to counter the increased expression of mdm2 and cyclin G1 and prolong the activity of p53 in response to DNA damage. Tob is also significantly upregulated after SM exposure. Tob is an antiproliferative gene, and mice lacking Tob are predisposed to cancer (*38*). Interestingly, decreased expression or altered phosphorylation of Tob has recently been shown to be an early event in lung cancer (*39*).

On the basis of the gene expression data that are summarized in Figure 4, it is reasonable to conclude that a single systemic exposure to SM results in damaged lung tissue DNA. The expression levels of p53 responsive genes are modulated across both dose and time as is displayed in Figure 3. Bax, EphA2, and IEX1/IER3 are important in regulating apoptotic pathways (*24*–*26*). ATF3, a transcription factor, is also upregulated by SM possibly for regulating various transcriptional processes in damaged cells (*28*). Snk is involved in the regulation of mitosis (*27*). Metallothionein is involved in the response to cellular injury (*29*).

In summary, this is the first oligonucleotide microarray analysis of lung tissue gene expression responses to a single systemic challenge with SM. The data reveal significant modulation of the expression levels of genes that are p53 responsive. This modulation of p53 responsive genes suggests that DNA damage is the most likely source of the initial injury. This damage subsequently results in modulation of genes that arrest cell cycle progression and enhance programmed cell death. Presumably, apoptotic activation in the face of extensive DNA damage may be intended to avert multiple mutations that could lead to cancer formation. Interestingly, it has been shown that there is a high incidence of lung cancer in former mustard gas workers and there are p53 mutations in many of these cancers (*40*–*44*). The data presented herein suggest the activation of initial mechanistic pathways of a carcinogenic/mutation event that has become associated with SM inhalation. This would require long-term studies to understand the progression of carcinogenesis resulting from SM exposure. In addition, these studies provide insight into potential therapeutic targets for treatment of SM exposure.

Acknowledgment. We thank Donald Fisher for research assistance, Richard Sweeney for help in information management, Robyn Lee for statistical support, D.J. Meyer and Tom Downey for data analysis assistance, and Albert Ruff and Gary Minsavage for critical reading of the manuscript.

Supporting Information Available: Tables of primers and probes used for Q-PCR analysis of selected genes, genes most significantly modulated by exposure to SM across all time points and doses examined, molecular functions most significantly affected by SM exposure, and biological processes most significantly affected by SM exposure. Figures of Q-PCR analysis of genes involved in cell cycle regulation from SM-exposed rat lung and PCA of gene expression data from SM-exposed rat lung. This material is available free of charge via the Internet at http://pubs.acs.org.

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